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# Inframolecular acid–base studies of the tris and tetrakis *myo*-inositol phosphates including the 1,2,3-trisphosphate motif

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**Abstract**

The intrinsic acid–base properties of the phosphate groups of three *myo*-inositol derivatives which display the 1,2,3-trisphosphate motif, i.e. (±)-*myo*-inositol 1,2,3-trisphosphate ($Ins(1,2,3)P_3$), (±)-*myo*-inositol 1,2,3,6-tetrakisphosphate ($Ins(1,2,3,6)P_4$), and (±)-*myo*-inositol 1,2,3,5-tetrakisphosphate ($Ins(1,2,3,5)P_4$) are reported. The studies were performed in 0.2 M KCl solution at 37 °C, near physiological ionic strength and temperature. In addition, in order to shed light on the transition metal complexation properties of $Ins(1,2,3)P_3$, the influence of the $Zn^{2+}$ cations on its $^{31}P$ NMR titration curves was investigated. From the titration curves as well as from the determined protonation microconstants, it appears that for $Ins(1,2,3)P_3$, the two lateral P1 and P3 phosphates strongly contribute to stabilise a proton on the central P2 phosphate. However, in the fully deprotonated form of $Ins(1,2,3)P_3$, P1 and P3 repulse each other so that they establish hydrogen bonds with, respectively, their neighbouring OH6 and OH4 hydroxyls. The 1,2,3-trisphosphate motif of $Ins(1,2,3,5)P_4$ behaves very similarly to that of $Ins(1,2,3)P_3$ indicating a poor interaction with the distant P5 phosphate. By contrast, moving a phosphate group from position 5 to position 6 on the *myo*-inositol ring as in $Ins(1,2,3,6)P_4$, leads to major changes in the basicity and cooperativity of the phosphate groups. Finally, the presence of $Zn^{2+}$ cations has a marked influence on the $^{31}P$ NMR titration curves of $Ins(1,2,3)P_3$, leading to the conclusion that two equatorial phosphates, assisted by a middle axial one, afford an optimal chelating moiety that is able to occupy all sites of the metal coordination polyhedron which could be the reason for its antioxidant properties.
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*Keywords:* Inositol phosphates; Inframolecular; NMR titration; Acid–base equilibria; Hydrogen bond

## 1. Introduction

Inositol phosphates make up a large but finite family of molecules, some members of which play key biological roles. The major representative of the latter is D-*myo*-inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) which is known for its signalling activities [1,2], but many other biologically active members have more recently attracted much interest [3–5]. It is thus possible to subdivide the family of inositol phosphates into two groups of compounds according to their biological relevance. While a wealth

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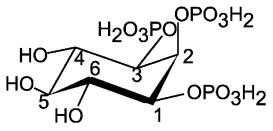
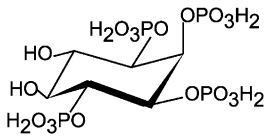
of studies has already been devoted to the group of compounds displaying intracellular functional properties, only little attention has been paid so far to the second group. However, inositol phosphates comprising a 1,2,3-trisphosphate grouping, which belong to this second group, showed interesting metal complexation properties which seem to be related to the *cis* 1,2,3 (equatorial–axial–equatorial) trisphosphate configuration. Spiers et al. first reported the synthesis and iron binding properties of *myo*-inositol 1,2,3-trisphosphate ($Ins(1,2,3)P_3$) [6,7] and demonstrated the ability of this ligand to inhibit $Fe^{3+}$-catalysed hydroxyl radical formation. The antioxidant property of *myo*-inositol 1,2,3-trisphosphate and *myo*-inositol 1,2,3,6-tetrakisphosphate ($Ins(1,2,3,6)P_4$) were confirmed [8], and the calcium absorption enhancing properties of $Ins(1,2,3,6)P_4$ were shown [9]. The intriguing 1,2,3-trisphosphate grouping requirement that accounts for the complexation and antioxidant potential of these ligands prompted us to apply our inframolecular approach to the study of their acid–base properties. This approach, previously applied to other inositol phosphates and analogues [10–15], relies on the determination of the intrinsic basicity of each phosphate group, which allows further insights into the cooperativity between the phosphate groups and the intramolecular interactions with the OH groups.

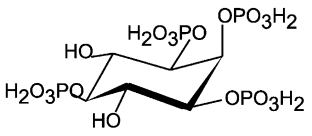
In this publication, we report the results obtained with three *myo*-inositol phosphates which display the 1,2,3-trisphosphate motif, i.e. (±)-*myo*-inositol 1,2,3-trisphosphate, (±)-*myo*-inositol 1,2,3,6-tetrakisphosphate[1] and (±)-*myo*-inositol 1,2,3,5-tetrakisphosphate ($Ins(1,2,3,5)P_4$). The studies were performed in 0.2 M KCl solution at 37 °C, near physiological ionic strength and temperature. Additionally, and in order to shed light on the transition metal complexation properties of $Ins(1,2,3)P_3$, the influence of $Zn^{2+}$ cations on its $^{31}P$ NMR titration curves was investigated. Due to its diamagnetic character, $Zn^{2+}$, in contrary to $Fe^{3+}$, makes it possible to carry out $^{31}P$ NMR titrations. The medium conditions used in those titrations were 25 °C and 0.1 M tetraethylammonium perchlorate ($Et_4NClO_4$) to minimise the interactions with the supporting electrolyte cations.




$Ins(1,2,3)P_3$




$Ins(1,2,3,6)P_4$




$Ins(1,2,3,5)P_4$

## 2. Experimental

*Materials*. For all the syntheses summarized below, *myo*-inositol was used as a starting material and the final products were obtained as cyclohexylammonium salts.

For the synthesis of *myo*-inositol 1,2,3,5-tetrakis-(phosphate), positions 1, 3 and 5 of *myo*-inositol were

[1] According to the nomenclature (±)-$Ins(1,2,3,6)P_4$ should be called (±)-$Ins(1,2,3,4)P_4$. However, since $Ins(1,2,3,6)P_4$ and $Ins(1,2,3,4)P_4$ are enantiomers and display the same acid–base properties, we use $Ins(1,2,3,6)P_4$ which is more frequently cited in the literature.

protected by a formiate group ($CH(OEt)_3$/-pTsOH/DMF) [16–20]. Positions 4 and 6 were then selectively benzylated (BnBr/NaH/imidazole/DMF) [16]. After deprotection of the formiate group in an acidic, methanolic solution (HCl/MeOH), the dibenzylated compound was phosphorylated with 2-diethyl-amino-1,2,3-benzodioxaphosphepan [21]. The final deprotection was performed by catalytic hydrogenation to yield *myo*-inositol 1,2,3,5-tetrakis(phosphate).

*myo*-Inositol 1,2,3,6-tetraskis(phosphate) was prepared from *myo*-inositol by protection of positions 1,2-3,4 with dicyclohexyl groups (1,1-diethyloxycyclohexane/pTsOH/DMF) [22] and further dibenzylation on positions 4 and 5. After deprotection of positions 1,2,3,4 in acidic conditions (MeOH/HCl), the dibenzylated compound was phosphorylated [21]. Debenzylation of positions 4 and 5 by catalytic hydrogenation yielded *myo*-inositol 1,2,3,6-tetrakis-(phosphate).

*myo*-Inositol 1,2,3-tris(phosphate) was synthetised from *myo*-inositol, protected with an orthoformate group on positions 1, 3 and 5 and benzyl groups on positions 2, 4 and 6. The tribenzylated orthoformate was opened by treatment with di-isobutylammonium hydride (DIBALH) to give the corresponding 1,3-acetal compound [23]. The resulting *meso*-2,4,6-tri-*O*-benzyl-1,3,-*O*-methylene *myo*-inositol was then benzylated on the free hydroxy group. Treatment with $TiCl_4$ in dichloromethane followed by methanolysis (MeOH/HCl) resulted deprotected positions 1, 2 and 3 [23], which were then phosphorylated. Catalytic dehydrogenation finally yielded *myo*-inositol 1,2,3-tris(phosphate).

Before the titrations, the ligands were converted into their acid form by ion exchange using an Amberlite IRN-77($H^+$) resin.

*Potentiometric studies and NMR determinations*. Potentiometric and NMR determinations were carried out as previously reported [10,11]. The experiments were performed in two steps in which the same initial solution of the studied compounds of about $3 \times 10^{-3}$ mol dm$^{-3}$ was successively subjected to potentiometric and $^{31}$P NMR or $^{1}$H NMR titrations. The processing of the pH measurements allowed the total concentration of the ligand and the acid to be determined. The NMR titrations were performed on 0.50 ml of solution in $^{2}H_2O$ on a Bruker DPX-300 Fourier transform spectrometer. One-dimensional $^{31}$P NMR spectra were recorded at 121.50 MHz and $^{31}$P chemical shifts values were referenced to an external 85% $H_3PO_4$ signal at 0.00 ppm with downfield shifts represented by positive values. Spectra were acquired over a spectral width of 10 ppm using a 0.1 s relaxation delay and a $\pi/2$ pulse. Typically 1K data points were sampled with a corresponding 0.4 s acquisition time. The spectra had a digital resolution of 1.19 Hz per point. The HypNMR and HYPERQUAD programs [24,25] were used to determine protonation constants. $^{1}$H NMR spectra were acquired with water presaturation over a spectral width of 6 ppm using a 3 s relaxation delay and a $\pi/2$ pulse. 4K data points were sampled with a corresponding 1.14 s acquisition time. The spectra had a digital resolution of 0.44 Hz per point. Data were zero-filled and a 1 Hz exponential line broadening function was applied prior to Fourier transformation. The temperature in both cases was controlled at 310.0 ± 0.5 K. The proton and phosphorus resonances of the studied compounds were assigned by performing proton–proton and phosphorus–proton 2D correlation experiments at two suitable pH values at least, thus allowing the titration curves to be unambiguously characterized.

## 3. Macroscopic and microscopic protonation constants

The studied inositol phosphates carry three or four phosphate groups, each group being able to bind one proton only for pHs ranging from 11 to 3. In this pH range, the protonation process, when defined step by step, may thus be quantified by $K_y$, characterising the equilibrium

$$H_{y-1}L^{(n-y)-} + H^+ \overset{K_y}{\Leftrightarrow} H_yL^{(n-y-1)-}$$

with $n = 7$ and $y = 1–3$ for inositol trisphosphates, and $n = 9$ and $y = 1–4$ for inositol tetrakisphosphates. It can be noted that log $K_y$ corresponds to the usual p$K_a$ value.

These constants, easily determined by potentiometric [26] or NMR titration methods [27], cannot be attributed to a given protonation site since most of them are separated by less than two log units;

therefore, a given macroscopic protonation step may involve several different basic sites.

The determination of the basicity of each individual phosphate group requires the entire resolution of a microprotonation scheme which, for instance for a trisfunctional molecule, takes eight microspecies and 12 related microconstants into account, as depicted in Fig. 1. In the case of Ins(1,2,3)$P_3$, due to the symmetry of the molecule, some protonation paths become equivalent, leading to a simpler protonation scheme (Table 1). The derivation of the corresponding microprotonation scheme for compounds carrying four phosphate groups is so far very difficult since 16 microspecies and 32 microconstants have to be envisaged.

For inositol phosphates, $^{31}$P NMR has proved to be a good method to study individual protonation [11–13,28–34], provided that the observed chemical shifts for the phosphorus resonances $\delta_i^{obs}$ mainly depend on the electronic effects accompanying the variations in the protonation states [11,13,14,35–38]. In that case, the protonated fraction $f_{i,p}$ of a phosphate group in position $i$ on the inositol phosphates can be calculated by:

$$f_{i,p} = \frac{\delta_i^{obs} - \delta_{i,d}}{\delta_{i,p} - \delta_{i,d}}$$

where $\delta_{i,p}$ and $\delta_{i,d}$ correspond, respectively, to the chemical shifts of the protonated and deprotonated fractions of the phosphates in position $i$. As previously shown, for trifunctional compounds, the individual protonation fractions $f_{i,p}$ may be expressed as a function of the macro- and micro-protonation constants, allowing the determination of the latter ones by non-linear regression. Thus, the microprotonation constants were calculated for Ins(1,2,3)$P_3$. At this stage of our investigations, microprotonation constants could not be obtained for tetraphosphorylated compounds, but the comparison of their $f_{i,p} = f(pH)$ curves allows qualitative inframolecular information to be drawn.

## 4. Results

*myo-Inositol* 1,2,3-*trisphosphate*. The $^{31}$P NMR titration curves for Ins(1,2,3)$P_3$, displayed in Fig. 2, show that, owing to the symmetry of the molecule, only two peaks appear. As expected, the resonances of the three phosphorus nuclei are shifted to highfield upon protonation. However, whereas the curve for the P1 and P3 phosphates appears almost monophasic and undergoes from their deprotonated ($\delta_d$) to their protonated ($\delta_p$) states with a chemical shift variation

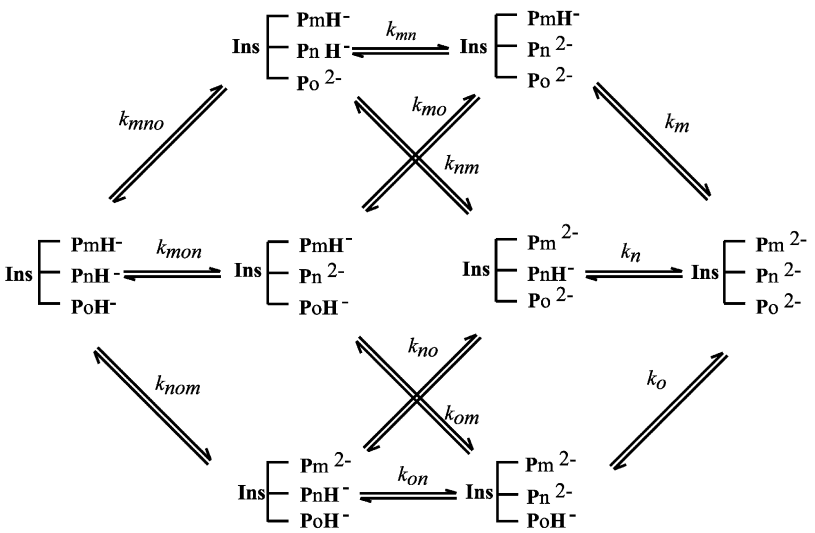



Fig. 1. Microprotonation scheme of an inositol phosphate carrying three phosphates at position *m*, *n* and *o*.

Table 1
Logarithms of the macro- and microprotonation constants for the studied compounds. log $k_i$, log $k_{ii'}$ and log $k_{ii'i''}$ represent a general designation for, respectively, the logarithms of the first, second, and third stepwise microprotonation constants. The uncertainties for the macroconstants, which are estimates of the standard deviations as calculated by Hyperquad are equal or less than 0.03. Those for the microconstants are equal or less than 0.15

| Ligand | $y$ | log $K_y$ | $i$ | log $k_i$ | $ii'$ | log $k_{ii'}$ | $ii'i''$ | log $k_{ii'i''}$ |
|---|---|---|---|---|---|---|---|---|
| Ins(1,2,3)$P_3$ | 1 | 9.37 | 1 or 3 | 8.67 | 12 and 32 | 7.78 | 123 and 321 | 6.04 |
| | 2 | 7.68 | 2 | 9.16 | 13 and 31 | 8.09 | 132 | 5.73 |
| | 3 | 5.43 | | | 21 and 23 | 7.30 | | |
| Ins(1,2,3,5)$P_4$ | 1 | 9.41 | | | | | | |
| | 2 | 7.79 | | | | | | |
| | 3 | 6.23 | | | | | | |
| | 4 | 5.50 | | | | | | |
| Ins(1,2,3,6)$P_4$ | 1 | 9.75 | | | | | | |
| | 2 | 8.18 | | | | | | |
| | 3 | 6.78 | | | | | | |
| | 4 | 5.56 | | | | | | |

of about $\Delta\delta = \delta_p - \delta_d = 5.43$ ppm, the P2 curve is clearly biphasic with a $\Delta\delta$ value limited to 3.17 ppm. For comparison purposes, in Fig. 2 we added the titration curve of *myo*-inositol 2,4,6-trisphosphate (Ins(2,4,6)$P_3$), whose phosphates are alternated on the *myo*-inositol ring. From these curves it clearly appears that in Ins(1,2,3)$P_3$ the two lateral P1 and P3 phosphates strongly contribute to the stabilisation of a proton on the central P2 phosphate. Thus, as can be seen in Table 1, the basicity of P2 is increased by 0.49 log units with respect to P1 and P3.

Fig. 3 shows the $^1$H NMR titration curves recorded at the same pH as for the phosphorus titration curves. It should first be mentioned that, for Ins(1,2,3)$P_3$ as well as for all the other compounds, from the coupling pattern and the values of the proton chemical shifts,

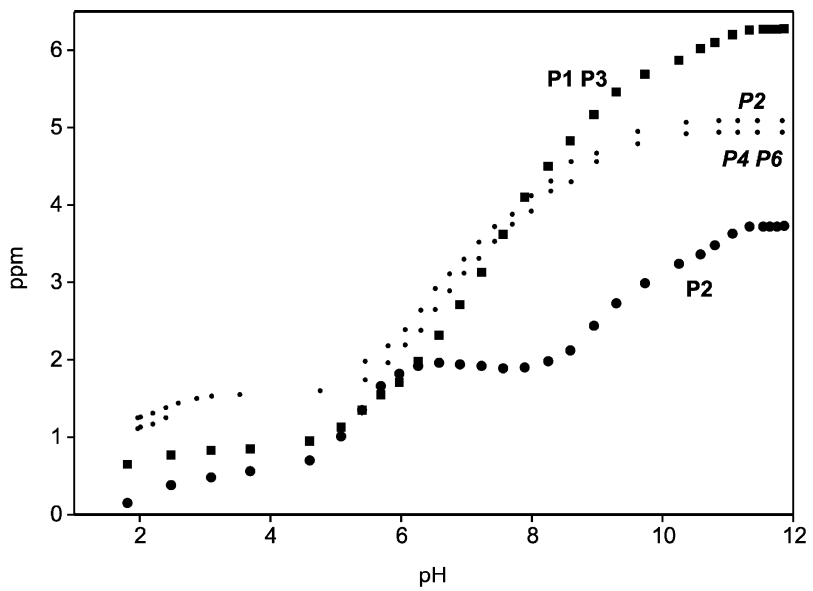



Fig. 2. Chemical shifts $\delta$ from $^{31}$P NMR titrations for Ins(1,2,3)$P_3$ as a function of pH in KCl 0.2 M at 37 °C ($D_2O$). For purpose of comparison the $\delta_i^{obs} = f(pH)$ for Ins(2,4,6)$P_3$ are superimposed (dotted lines).

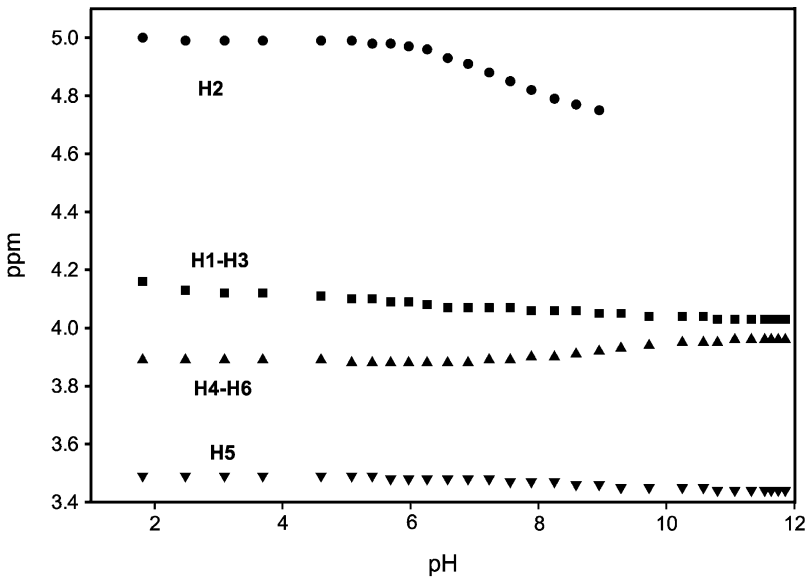



Fig. 3. Chemical shifts $\delta$ from a $^{1}$H NMR titration for Ins(1,2,3)$P_3$ as a function of pH in 0.2 M KCl at 37 °C ($D_2O$).

there is no evidence of any ring flip. It can therefore be considered that the *cis* 1,2,3 (equatorial–axial–equatorial) trisphosphate configuration remains over all the studied pH range. Upon protonation of the three phosphates the H2 signal moves downfields from pH 9, where it is first observed, to pH 6. The H4–H6 resonances are shifted slightly to highfield and the remaining H1–H3 and H5 chemical shifts almost remain constant.

*myo-Inositol* 1,2,3,5-*tetrakisphosphate*. The phosphorus NMR titration curves of the vicinal P1, P2 and P3 phosphates (Fig. 4) closely resemble those of Ins(1,2,3)$P_3$. Fig. 4 also displays the curves of *myo*-inositol 1,5-bisphosphate (Ins(1,5)$P_2$), which show that the monophasic P5 signal of Ins(1,5)$P_2$ superimposes well with the corresponding resonance of Ins(1,2,3,5)$P_4$. This is the sign of poor interaction between the 1,2,3-trisphosphate motif and the distant P5 phosphate. Therefore, there is a very slight gain in basicity of the three vicinal phosphates once the fourth P5 phosphate is added (Table 1).

*myo-Inositol* 1,2,3,6-*tetrakisphosphate*. The $^{31}$P NMR titration curves of Ins(1,2,3,5)$P_4$ show (Fig. 5) four distinct resonances, two of which being nearly monophasic (P3 and P6) and two being biphasic (P1 and P2). Also, with regard to the previous compounds, P1 and P3 behave very differently, both as for the shape of their curves, their $\Delta\delta$ values, as well as their intrinsic basicity. Thus, comparison of the $f_{i,p} = f(\text{pH})$ curves for P1 and P3 (curves not shown) indicates the higher basicity of the former phosphate group. For Ins(1,2,3,6)$P_4$ all proton resonances are satisfactorily resolved over a wide part of the studied pH range (Fig. 6). By decreasing the pH, H1, H2, H3 and, to a lesser extent, H5 shift to lower fields simultaneously with the protonation of the phosphate groups. H6 shows a more complicated pattern, with first a slight shift highfield followed by a shift in the opposite direction. Finally, H4 also experiences a highfield shift first, and then remains constant.

*myo-Inositol* 1,2,3-*trisphosphate–*$Zn^{2+}$ *system*. In contrast to the previous titrations, the curves reported in Fig. 7 were obtained in 0.1 M $Et_4NClO_4$ solutions, i.e. in the absence of potassium cations which would have strongly interfered with $Zn^{2+}$ in the complexation reaction. By considering the curves corresponding to an absence of zinc cations (full lines), it can be seen that, with regard to Fig. 2, these curves are, in most of the pH range, shifted by about 1.5 log unit to higher pH values. This clearly indicates a strong competition between the protons and potassium cations for the binding to the phosphate group.

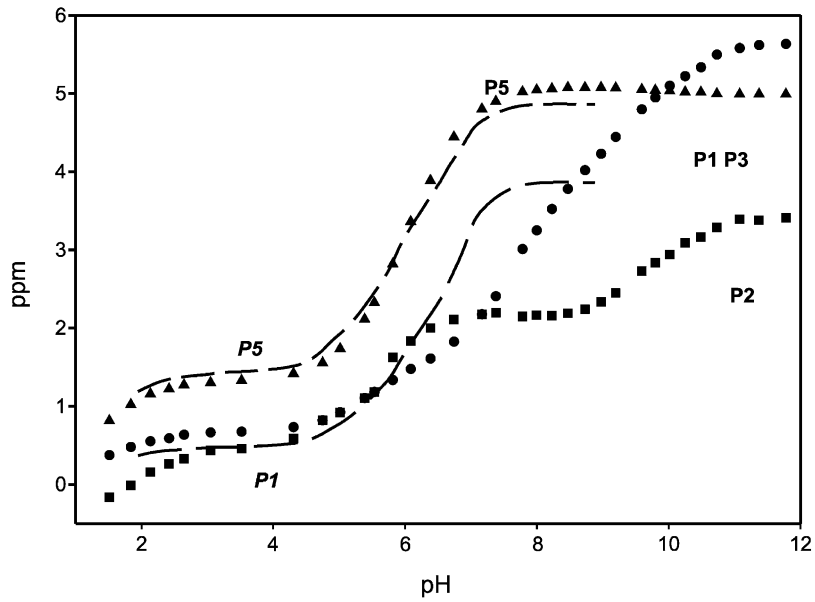



Fig. 4. Chemical shifts $\delta$ from $^{31}$P NMR titrations for Ins(1,2,3,5)$P_4$ as a function of pH in KCl 0.2 M at 37 °C ($D_2O$). For purpose of comparison the $\delta_i^{obs} = f(pH)$ for Ins(1,5)$P_2$ are superimposed (broken lines).

Remarkably, $Zn^{2+}$ cations still displace the curves to lower pH (point curves), but also markedly modify the shape of the curves. For instance, the P1–P3 curves, while monophasic in the absence of $Zn^{2+}$ cations, become biphasic in their presence.

## 5. Discussion

Upon protonation of a phosphate group, the phosphorus nuclei usually undergo a chemical shift variation of about 3.8–4.0 ppm towards higher fields.

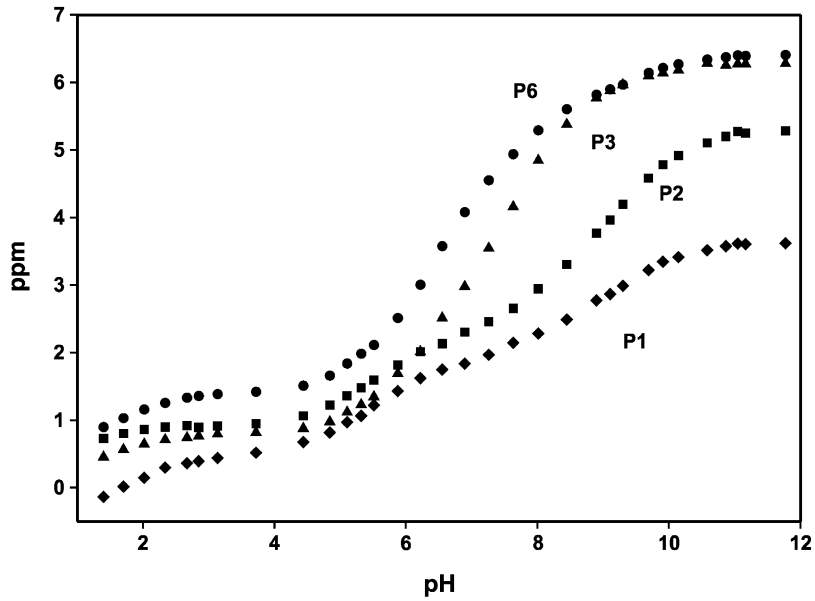



Fig. 5. Chemical shifts $\delta$ from $^{31}$P NMR titrations for Ins(1,2,3,6)$P_4$ as a function of pH in KCl 0.2 M at 37 °C ($D_2O$).

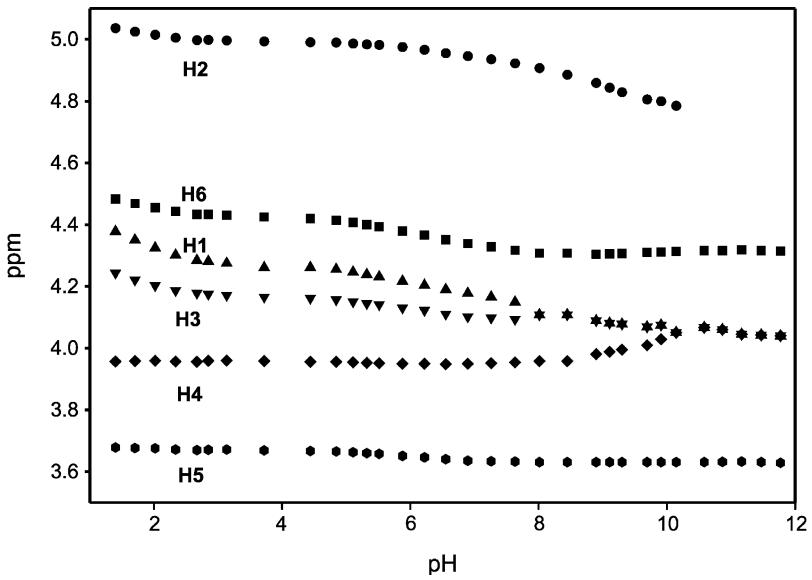



Fig. 6. Chemical shifts $\delta$ from a $^{1}$H NMR titration for Ins(1,2,3,6)$P_4$ as a function of pH in 0.2 M KCl at 37 °C ($D_2O$).

Surprisingly, the P1P3 and P2 resonances variations of Ins(1,2,3)$P_3$ are, respectively, much larger (5.4) and much smaller (3.2) than those of an ordinary ester phosphate. This is the result of unusual $\delta_p$ and $\delta_d$ values of the three phosphates, as can be seen, for instance, on the P2 signals of Ins(1,2,3)$P_3$ and Ins(2,4,6)$P_3$ in Fig. 2. Previous studies [12–14,34,39–41] showed that, for a given protonation state,

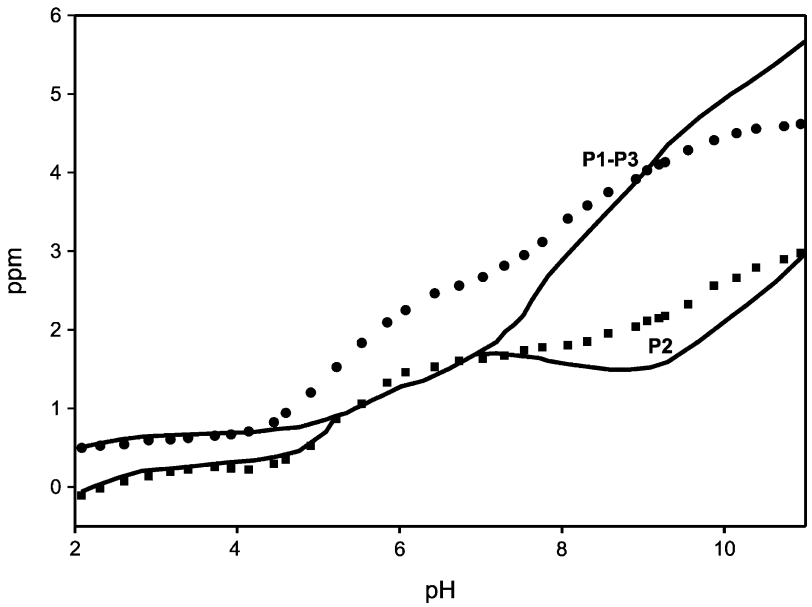



Fig. 7. Chemical shifts $\delta$ from $^{31}$P NMR titrations for Ins(1,2,3)$P_3$ in the presence (points) and the absence (lines) of $Zn^{2+}$ as a function of pH in $Et_4NClO_4$ 0.1 M at 25 °C (10% $D_2O$).

the chemical shifts of the phosphates mainly depend on the involvement of the phosphate group in hydrogen bonds with either vicinal hydroxyl groups or water molecules. Thus a local hydrophilic pocket drives the chemical shifts towards lower fields, whereas the opposite occurs when the local dielectric constant of the medium decreases. It is therefore expected that, in Ins(1,2,3)$P_3$, P2 experiences a less polar environment than P1P3. An explanation for these observations may be proposed by also considering the $^1$H NMR titration curves (Fig. 3). Before discussing these curves, it must be recalled that in the most general case, binding of a proton to a basic site leads to an electron density decrease and thus, via a through-bond effect, to a shift of the proton resonances to lower fields. An opposite trend, called 'wrongway shift' has already been observed in nucleotides [42–46], inositol phosphates [13,15] and natural compounds such as adenophostin A [47]. It occurs when a highly negatively charged phosphate group approaches a hydrogen atom. This electrostatic effect operates through the field [45,47] and affects the chemical shifts of both the hydrogen and phosphorus atoms, thus providing valuable structural and conformational information.

Clearly, the H4–H6 protons undergo a wrongway shift simultaneously to the protonation of the P2 phosphate, i.e. from pH 12 to 8. According to the Karplus/Altona [48] equation, the value of $^3J_{H-P}$ P2 coupling constant (9.2 Hz at pH 11.5) indicates a free rotation of the P2 phosphate group. Therefore, the close approach of P2 to the axial H4 and H6 hydrogens enables their interaction and thus affects their chemical shifts. On the other hand, the axial orientation of P2 does not hinder the electrostatic repulsion between the P1 and P3 phosphates, which tends to decrease while they get protonated. When carrying two negative charges, P1 and P3 repulse each other so much that they establish hydrogen bonds with, respectively, their neighbouring OH6 and OH4 hydroxyls with the consequence of chemical shifts displaced to lower fields. Interestingly, although few crystal structures of inositol phosphates are available, that of Ins(1,2,3)$P_3$ does exist. In the last structure published, Spiers et al. [49] observe a strong hydrogen bond between the protons carried by the P1 phosphate and the deprotonated P2 phosphate, which is in line with the strong cooperativity between both phosphates mentioned in our studies. However, according to the higher basicity of P2 compared to P1, the proton is most likely carried by P2 in aqueous solution. Due to the huge importance of hydration, it is not surprising that Ins(1,2,3)$P_3$ behaves differently in the solid state than in solution. Nevertheless, the expected hydrogen bonds between the equatorial P1 and P3 phosphates and their vicinal hydroxyl groups are likely to exist in solution since a bond of this type between the charged oxygen atom of the P3 phosphate and OH4 was observed in the crystal structure.

As shown in Fig. 4, the case of Ins(1,2,3,5)$P_4$ does not differ from that of Ins(1,2,3)$P_3$, the monophasic P5 curve being only added to those of Ins(1,2,3)$P_3$. Noteworthy is that P5 of Ins(1,2,3,5)$P_4$ superimposes with P5 of Ins(1,5)$P_2$, whereas their respective P1 curves differ largely. This again stresses the unusual behaviour of the 1,2,3-trisphosphate grouping in the studied compounds.

From Ins(1,2,3,5)$P_4$ to Ins(1,2,3,6)$P_4$, a phosphate group moved from position 5 to position 6 on the *myo*-inositol ring, leading to major changes in the basicity and cooperativity of phosphate groups, as shown in Fig. 5. In particular in Ins(1,2,3,6)$P_4$, P1 and P3 markedly differentiate since P1 becomes biphasic and has a $\delta_p$ value by 2.0 ppm lower than that of Ins(1,2,3,5)$P_4$. This may be attributed to the presence of the P6 phosphate group which, at high pHs, repels P1 to minimise charge interactions and brings it in close proximity to H2 while contributing at lower pHs to stabilise a proton in between both phosphates. Also, P2 $\delta_p$ value increases from 3.4 ppm in Ins(1,2,3,5)$P_4$ to 5.3 ppm in Ins(1,2,3,6)$P_4$. In the latter case, presumably, the access of the phosphate P2 to H4 and H6 is hindered by the presence of the P6 phosphate: thus the axial phosphate group experiences a less hydrophobic environment, which leads to a more usual $\delta_p$ value.

As mentioned previously, inositol phosphates which include a 1,2,3-trisphosphate grouping presumably display antioxidant properties due to the inhibition of $Fe^{3+}$-catalysed $HO^{\cdot}$ formation [6–8]. Among the three studied compounds, Spiers et al. [7] showed that Ins(1,2,3)$P_3$ and Ins(1,2,3,5)$P_4$ are the most effective ones in the suppression of $HO^{\cdot}$ generation. Both compounds also behave very similarly at an inframolecular level. It would have been interesting to investigate the coordination status

of iron in the $Fe^{3+}$–Ins(1,2,3)$P_3$ system, but the iron paramagneticity prevents the use of $^{31}P$ NMR techniques. Thus the effect of $Zn^{2+}$ cations on the titration curves was considered, rather than that of $Fe^{3+}$ cations. Fig. 7 shows that the P1P3 phosphates are much more affected by $Zn^{2+}$ than the P2 phosphate. The two equatorial phosphates bind $Zn^{2+}$ in the 4.3–11 pH range, whereas phosphate P2 only seems to be involved in the complexation from pH 7 upwards. Furthermore, the $\delta_d$ value for P1P3 decreases by about 1 ppm, indicating that, at high pH, coordination to $Zn^{2+}$ largely competes with the formation of hydrogen bonds with vicinal hydroxyl groups. Preliminary results on the stability constants of the $Fe^{3+}$–Ins(1,2,3)$P_3$ and $Zn^{2+}$–Ins(1,2,3)$P_3$ systems (unpublished data) show that these complexes are equally or even less stable than those of Ins(1,2,6)$P_3$ [50], although they are more effective in the inhibition of free radical formation. Thus the mode of coordination of the metallic cation seems more important than the stability of the complexes. From our studies it appears that two equatorial phosphates, assisted by a middle axial one, afford an optimal chelating moiety that is able to occupy all sites of the metal coordination polyhedron.

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